Author's response to reviews

Title: The Autophagy GABARAPL1 gene is Epigenetically Regulated in Breast Cancer Models.

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Author's response to reviews: see over
Dear Editor and Reviewers,

First, we would like to thank the reviewers for their helpful comments which have helped us improve our manuscript. All the modifications included in this revised version of our manuscript have been highlighted in red.

Reviewer 1:

Hervouet et al demonstrated that a specific decrease of GABARAPL1 expression was associated with both DNA methylation and histone deacetylation and that CREB-1 recruitment on GABARAPL1 promoter was required for GABARAPL1 expression. In general, the experiments were well planned and data well presented.

Minor concerns:
1. Line 260: Loss of global DNA methylation in several tissues (Fig. 2A). It is unclear how Fig. 2A illustrates this point.

Answer: we agree with reviewer 1 that our sentence maybe misleading. Our data presented in Fig. 2A showed that the global amount of methylated DNA is similar in the different control DNA samples (NT) but presents a higher variability in Grade III tumor samples. Indeed, we observed that the level of methylated DNA is similar to the one observed in control samples (8 out of 13 samples), increased (1 out of 13) or decreased (4 out of 13). These observations are consistent with previous publications which reported that global DNA methylation is frequently impaired in solid tumors. This point has been clarified in the RESULTS section (line 250-260) and two additional references have been added.

2. Line 333: “Although our results suggest that GABARAPL1 protein expression was also increased in MDA-MB-334 453 cells, the GABARAPL1 content was too low to be quantified.” This is confusing. How can the results suggest an increase when it was too low to be quantified?
Answer: we apologize for this confusing sentence. Unlike MCF-7 cells which presented a clear increase of GABARAPL1 expression following treatment with TSA, only a weak and diffuse signal was detected in MDA-MB-453 cells treated with TSA, even after a long exposure, making its quantification difficult and the conclusion uncertain. We modified the text accordingly in the RESULTS section.

3. BECN-1 rather than BECLIN-1

Answer: “BECLIN-1” has been replaced by “BECN-1” throughout the manuscript and this abbreviation has been added in the ABBREVIATIONS section.

4. There are a few places where the English usage was unusual and needs attention and/or clarification. For example:
   a. Line 50: organites -- organelles.
   b. Line 54: even if it is currently admitted—even though evidence indicated
   c. Line 78: it is now admitted—it is now recognized.

Answer: we thank reviewer 1 for these suggestions. The corrections have been made accordingly.

Level of interest: An article of outstanding merit and interest in its field
Quality of written English: Needs some language corrections before being published
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests: no competing interest

Reviewer 2:
The manuscript, entitled “The Autophagy GABARAPL1 gene is Epigenetically Regulated in Breast Cancer Models”, by Hervouet et al demonstrated that (1) A specific decrease of GABARAPL1 expression in breast cancers was associated with both DNA methylation and histone deacetylation; (2) CREB-1 recruitment on GABARAPL1 promoter was required for GABARAPL1 expression. The authors concluded that epigenetic inhibitors and CREB-1
modulators may be used in the future to regulate autophagy in breast cancer cells. The manuscript was well written. Most experiments were straightforward. The experimental findings were in general supportive of the authors’ conclusions. For the most part, the data interpretations were appropriate. Nonetheless, the authors do not provide enough data regarding the phenotypes of autophagy associated with the reported observations. The methods used to demonstrate the phenotypes of autophagy in human breast cancer models are limited. The manuscript can be further improved if the following concerns can be effectively addressed:

Major Compulsory Revisions:
The authors should perform additional experiments to explore the possible associations between DNA methylation-related low expression levels of GABARAPL1 proteins and autophagy phenotypes in human breast cancer tissues and cell lines.

Answer: the purpose of the present study was to determine whether the expression of GABARAPL1 gene was regulated by epigenetic modifications in breast cancer since its expression has been previously described to be down-regulated in these tumors [1]. We also previously reported that knockdown of GABARAPL1 in a breast cancer cell line dramatically impaired autophagy levels [1] whereas another study demonstrated that overexpression of this protein decreased breast cancer cell proliferation [2]. Nevertheless, no current data clearly established a link between these two GABARAPL1-driven effects. We therefore totally agree with reviewer 2 that understanding the links between epigenetic regulation of GABARAPL1, autophagy and breast cancer is an important question. However this point cannot be easily assessed and we think it is beyond the scope of this study and will require further experiments.

Taken together, these former published data and the ones presented in this study suggest that the restoration of GABARAPL1 expression using epigenetic drugs in breast cancer might regulate autophagy levels as well as cancer cell proliferation and as a consequence help to limit aggressiveness of these tumors. However, these compounds are not specific of GABARAPL1 gene and the evaluation of their role on specific GABARAPL1-mediated autophagy remains difficult in a cell culture or tissue environment. Nevertheless, to address the question of reviewer 2, we included a new supplementary Figure 2. This new set of data
showed that in MCF-7 cells treated with 5-aza-CdR/TSA, we observed an accumulation of the autophagosome-associated LC3B form (LC3B-II) (Supp Fig. 2A). We also quantified an increase of the number of cells presenting GFP-LC3 positive puncta in MCF-7 cells transfected with pGFP-LC3 cells and treated with TSA compared to untreated cells (Supp Fig. 2B). This induction of autophagy might be partially explained by the restoration of GABARAPL1 expression in these cells which do not express detectable level of the protein before treatment but we cannot exclude that these compounds regulate other gene methylation status or protein acetylation (histones or cytosolic proteins) involved in the autophagy pathway. For example, Zou et al also recently reported that the re-expression of the ARHI protein following treatment with HDACi and DNMTi in breast cancer cells induced autophagy [3] and a recent study describes that the acetylation status of LC3B regulates its conjugation to autophagosomes and autophagy [4]. We also showed that treatment of MCF-7 cells with 5-aza-CdR/TSA decreased cell proliferation and clonogenecity (Supp Fig. 2C and D) but, once more, we cannot conclude that these effects are GABARAPL1-linked since the inhibitors used are not specific of this gene. In conclusion, all these data showed that 5-aza-CdR/TSA treatment can restore GABARAPL1 expression, increase autophagy levels and decrease cell proliferation but the link between these different effects needs to be confirmed by future experiments and will require specific inhibitors.

The description of this new Supp Fig. 2 has been included in the RESULTS section.


Minor Essential Revisions:
1. Page 6 (140): consents;
2. Page 20 (486): helped draft;

Answer: we thank the reviewer for these suggestions. The corrections have been made accordingly.

Level of interest: An article of outstanding merit and interest in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests: I declare that I have no competing interests.